



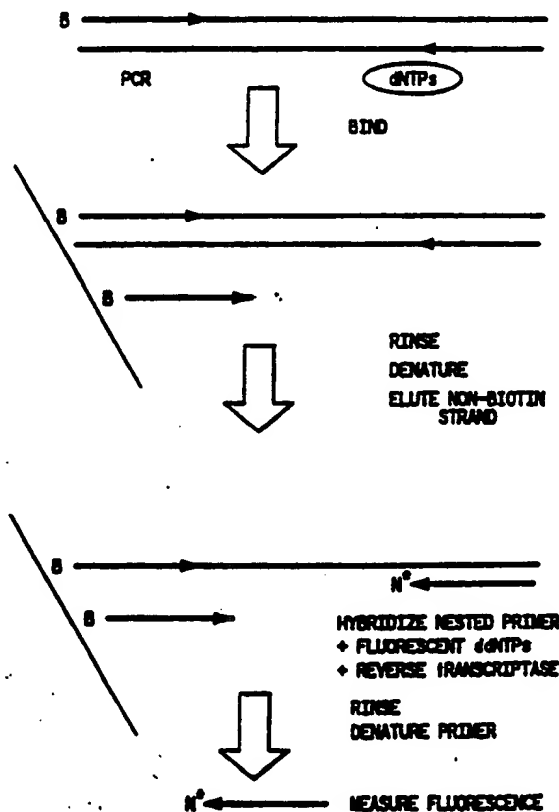
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(21) International Application Number: PCT/US92/01691 (22) International Filing Date: 12 March 1992 (12.03.92) (30) Priority data: 669,568 13 March 1991 (13.03.91) US (60) Parent Application or Grant (63) Related by Continuation US 669,568 (CIP) Filed on 13 March 1991 (13.03.91) (71) Applicant (for all designated States except US): E.I. DU PONT DE NEMOURS AND COMPANY [US/US]; 1007 Market Street, Wilmington, DE 19898 (US).	(72) Inventors; and (75) Inventors/Applicants (for US only) : LIVAK, Kenneth, J. [US/US]; 2418 Shellpot Drive, Wilmington, DE 19803 (US). RAFALSKI, Jan, Antoni [US/US]; 2028 Longcome Drive, Wilmington, DE 19810 (US). SHEPHERD, Nancy, Faye, Stacy [US/US]; 210 Partridge Way, Kennett Square, PA 19348 (US). (74) Agent: GALLEGOS, R., Thomas; E.I. du Pont de Nemours and Company, Legal/Patent Records Center, 1007 Market Street, Wilmington, DE 19898 (US). (81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), SE (European patent), US. Published With international search report.	

(54) Title: METHOD OF IDENTIFYING A NUCLEOTIDE PRESENT AT A DEFINED POSITION IN A NUCLEIC ACID

(57) Abstract

A method is described for identifying a nucleotide at a defined point on a nucleic acid sequence. An oligonucleotide probe is annealed to a target nucleotide sequence of the nucleic acid sample at a point immediately adjacent and 3' to the nucleotide of interest. The probe is then extended in the direction of the nucleotide of interest in a reaction medium containing at least one chain terminating nucleotide triphosphate (ATP, GTP, TTP and CTP). The nucleotide of interest is complementary to the labeled nucleotide incorporated into the primer by the extension reaction.



TITLEMETHOD OF IDENTIFYING A NUCLEOTIDE PRESENT
AT A DEFINED POSITION IN A NUCLEIC ACID5 FIELD OF THE INVENTION

This invention relates to a rapid, convenient process to identify a nucleotide present at a specific position in a nucleic acid chain (DNA or RNA) of a biological sample.

10 BACKGROUND OF THE INVENTION

The nucleic acid content of any organism is the essence of that organism, and differences in the nucleic acid are known to be of primary importance in distinguishing one from another. The science of genetics is based on the identification and characterization of differences in nucleic acid sequence. These differences, or polymorphisms, are often termed "mutations" and may be due to nucleotide substitution, insertion or deletion. Thus, many techniques have been developed to compare homologous segments of DNA or RNA to determine if the segments are identical or if they differ at one or more nucleotides. Identification of genetic polymorphisms is useful for genetic diagnoses in medicine, identification of individuals in forensic science, identification of pathogenic organisms, construction of genetic polymorphism maps for locating genes important in disease and in agriculture and for breeding of plants and animals.

30 The most definitive method for comparing DNA segments is to determine the complete nucleotide sequence of each segment. Examples of how sequencing has been used to study mutations in human genes are included in the publications of Engelke, et. al., Proc.

detection in a genomic DNA sample is very labor intensive for it requires preliminary steps of genomic DNA isolation, restriction, gel electrophoresis and Southern transfer steps, before hybridization to a probe that is generally radioactively labeled for sensitive detection of homologous sequences. A major problem associated with RFLP detection is the necessity of the polymorphism to affect cleavage with a restriction endonuclease, therefore many mutations cannot be detected with this method (Jeffreys, Cell 18:1-18, 1979). More importantly, although RFLP and several other methods in the prior art (e.g. Wallace et al., Nucl. Acids Res. 9:879-894, 1981 or Saiki, et al., U.S. Pat. No. 4,683,194 or Kornher et al., U.S. Pat. No. 4,879,214) are useful for finding polymorphisms in DNA, they do not elucidate the exact nature of the nucleotide present at a specific position on the nucleic acid sequence. In some applications, such as prenatal diagnosis, knowledge of which nucleotide is present at a given position is extremely important, since some nucleotide changes do not alter the coding capacity of a gene and are therefore "silent" with respect to phenotype. Those techniques that elucidate the nature of the nucleotide present are discussed below.

Many techniques designed to elucidate the nature of a nucleic acid polymorphism involve hybridization with a polynucleotide probe, a portion of which is complementary to the nucleotide position(s) of interest. A target sequence that is perfectly complementary to the probe can be distinguished from a target that differs by as little as a single nucleotide in a variety of ways. A technique involving amplification and mismatch detection (AMD), described by Montandon et al., Nucl. Acids Res. 17:3347-3358, 1989, utilizes amplification of

Amplification Reaction (LAR) as reported by Wu and Wallace, Genomics 4:560-569, (1989) and Wallace and Skolnick (WO 89/10414) is also dependent upon ligation of oligonucleotides whose 3-prime ends include the nucleotide position of interest. They demonstrate that four pairs of oligonucleotides that are complementary to the upper and lower strand of the target DNA will be exponentially amplified only if there is perfect complementarity between the oligonucleotides and the target DNA. The patent of Vary et al., U.S. Pat. No. 4,851,331, also depends upon an enzymatic reaction that requires one end of the oligonucleotide probe to form a perfect, complementary matched basepair with the target nucleotide sequence. As in the examples above, an oligonucleotide probe is designed such that the 3-prime end of the complementary probe includes the specific nucleotide position of interest. After annealing this oligonucleotide probe to the template DNA, a polymerase that replicates nucleic acid strands in a template directed fashion is used to incorporate modified nucleotides into a newly synthesized strand. If the 3-prime end of the oligonucleotide probe did not contain a nucleotide complementary to the target nucleotide sequence, then the polymerase cannot begin the replication process. The amount of incorporation is a measure of the amount of the specific template DNA in the biological sample. This same principle of utilizing a polymerase to discriminate whether there is a mismatched base at the 3-prime end of the primer was also recently combined with the PCR to give an exponential rather than linear increase of the reaction products in a process called Allele-specific Polymerase Chain Reaction (ASPCR) (Wu et al., Proc. Natl. Acad. Sci. 86:2757-2760, 1989). In their example, the

same result can occur if the assay method is too sensitive such that even inefficient ligation or replication is detected as a positive signal.

- Misincorporation of nucleotide substrate, well
- 5 documented in the literature for polymerases; Ricchetti and Buc, *The EMBO J.* 9:1583-1593, (1990); or template-independent ligation products due to blunt end ligation; Hayashi et al., *Nucl. Acids Res.* 14:7617-7631, (1986);
- 10 can lead to a false signal if not adequately suppressed in the reaction. Such misincorporation is especially apparent when the correct, complementary nucleotide substrate is absent from the reaction. The polymerase chain reaction is quite dependent upon products generated during the first few rounds of amplification.
- 15 The difficulty of devising conditions that totally suppress amplification by the primer that contains a mismatched base at the 3' end is also documented in Chehab and Kan; *Proc. Natl. Acad. Sci.* 86:9181 (1989); where fluorescence values as high as 0.8 were considered
- 20 negative for they were less than 1.0, while all values above 1.0 were considered positive for amplification (even values as low as 1.4 were considered positive).

In the technique described in Mundy, U.S. Pat. No. 4,656,127, specific mutations can be detected by first

25 hybridizing a labeled DNA probe to the target nucleic acid in order to form a hybrid in which the 3' end of the probe is positioned adjacent to the specific base being analyzed. Then, a DNA polymerase is used to add a nucleotide analog, such as a thionucleotide, to the

30 probe strand, but only if the analog is complementary to the specific base being analyzed. Finally, the probe-target hybrid is treated with exonuclease III. If the nucleotide analog has been incorporated, the labeled probe is protected from nuclease digestion. Absence of

The present invention solves several problems inherent in the Sokolov method. (1) This invention is not dependent upon radioactive substrates nor the time-consuming monitoring of the assay via a polyacrylamide gel and subsequent autoradiography. (2) This invention uses chain-terminating nucleotides as substrates in the reaction, therefore preventing incorporation of several of the same nucleotide in the primer extension product if there are several of the same nucleotides present in a row on the template. (3) The analysis of Sokolov required four separate reactions whereas the present invention would need only one reaction to gain the same amount of information. (4) As mentioned above, if the correct, complementary nucleotide substrate is not present in the reaction, then significant misincorporation can occur in the Sokolov reaction. Misincorporation is substantially prevented in the present invention.

Automation of the ASPCR reaction was described but not demonstrated in Wu et al., Proc. Natl. Acad. Sci. 86:2757-2760, (1989), and again by Chehab and Kan, Proc. Natl. Acad. Sci. 86:9178-9182, (1989), for fluorescent ASPCR. In both cases, each ASPCR reaction is performed using one biotin-labeled primer and one fluorescently-labeled primer. The biotinylated, double-stranded amplification products are then separated from unincorporated fluorescent primer using streptavidin coated magnetic beads. The color of the amplified DNA would then be determined fluorometricly through a fiber optic bundle, or alternatively, by separation and detection on a sequencing gel as is currently performed for DNA sequencing using fluorescently labeled primers. The differences between this method and that of the present invention are significant. Most importantly,

to automation due to the lack of centrifugation steps and the ability to quickly assay reaction products without a gel separation step. If a gel separation assay is used, then multiplexing of samples based on
5 differences in length of the probe oligonucleotide is possible. Alternatively, the probe oligonucleotide of several reactions can be of the same length, but loaded at different times after pausing the electrophoresis run.

10

SUMMARY OF THE INVENTION

The present invention provides a process for identifying the nucleotide present at a specific position in a nucleic acid sequence. It is based upon the selective attachment of one of four chain-
15 terminating nucleotides, that are detectably labeled and distinguishable, onto a probe in a complementary, template dependent fashion. The probe is designed to selectively hybridize to a target nucleotide sequence and oriented such that a one nucleotide extension of the
20 probe, usually in the 3-prime direction, will base pair to the nucleotide position of interest. The oligonucleotide probe, the nucleic acid containing the target nucleotide sequence, or both, may contain a site for specific immobilization to facilitate separation
25 from unincorporated nucleotides and primers, such that the labeled nucleotides incorporated into the reaction product can be measured without use of a gel system such as agarose or acrylamide.

Thus the present invention provides a method for the
30 identification of the nucleotide present at a single, defined position in the nucleic acid which comprises the following steps:

(a) contacting a nucleic acid analyte with a probe oligonucleotide of sufficient length and appropriate

DESCRIPTION OF FIGURES

Figure 1, comprising Figures 1a-1h, illustrate in various schematic forms, the location of various components of the process of this invention.

5 Figure 1a illustrates an analyte strand (An) which contains the nucleotide position of interest (N), the identity of which is to be determined by the assay. A target nucleotide sequence (TNS) immediately 3' of, but not including the nucleotide position of interest is
10 illustrated. A double strand nucleic acid region forms when a probe binds to analyte strand An by complementary base pairing to the target nucleotide sequence TNS.

 Figure 1b illustrates the incorporation of a chain
15 terminating nucleotide (N*) complementary to the nucleotide of interest (N) after contacting the double stranded region in Figure 1a with a polymerase capable of primer extension. (The * in this and subsequent
 figures is used to illustrate a detectable label attached to the nucleotide).

20 Figure 1c illustrates the same features as Figure 1a, but with a specific example showing the nucleotide of interest as a thymidine (T).

 Figure 1d illustrates the same features as Figure
25 1b, but using the same specific example as Figure 1c, to show the result of enzymatic incorporation of a detectably labeled adenosine at the 3' terminus of the probe as the nucleotide complementary to the nucleotide of interest (thymidine).

 Figure 1e illustrates the incorporation of a
30 detectably labeled guanosine at the 3' terminus of the probe and complementary to the nucleotide of interest (cytidine).

 Figure 1f illustrates the use of another analyte strand for the assay (the complementary strand of the

examples are detected with the Genesis 2000 DNA analysis system.

Figure 7a illustrates the output signal [ratio of the green line to red line peak height, +/- one standard deviation] of data obtained as in Figure 7b and 7c for each of the four detectably labeled nucleotides used in the examples detected either through a gel or through a capillary.

Figure 7b is representative data showing the position and relative peak heights of the two photomultiplier tube signals (red and green lines) when SF-ddGTP-505 or SF-ddTTP-526 are electrophoresed through a urea-polyacrylamide slab gel mounted on the Genesis 2000.

Figure 7c illustrates the output signal obtained when SF-ddGTP-505 or SF-ddCTP-519 are each passed four times (therefore four peaks) through an empty capillary mounted for detection on the Genesis 2000 unit.

Figure 8 illustrates the double stranded portion of the mouse RNA polymerase II gene that was amplified using PCR primer 1 and PCR primer 2, as well as the position and sequence of the various oligonucleotide probes used in Examples 1-5.

Figure 9 illustrates the sequence of the Wildtype and the Mutant allele of the RNA polymerase II gene between nucleotides 5395 and 5454, with the difference between the two alleles indicated by boldface type at position 5430.

Figure 10 illustrates the data obtained in Example 1: Incorporation of either labeled SF-ddATP-512, SF-ddGTP-505, or both in approximately equal amounts, when probe A is used on nucleic acid samples known to be either Wildtype, Mutant, or Heterozygous at nucleotide position 5430 of the RNA polymerase II gene.

biological sample with respect to specific, nucleic acid sequence information (e.g. nucleotide positions correlated with phenotypic differences among individuals between species or in tissues). Single base pair mutations such as transitions, transversions, insertion, deletion as well as more complex rearrangements can be assayed using the method of the present invention if the appropriate oligonucleotide probe is designed (Figure 5).

10 The presence of a target nucleic acid in a biological sample may be detected generally as the presence or absence of an incorporated nucleotide. Individual nucleotides located at selected sites in the nucleic acid sample may also be identified. The method
15 presented here is generally applicable to all nucleic acid sequences (DNA or RNA), whether they are single or double stranded, as long as the target nucleic acid strand is of sufficient length to form a hybrid with a complementary, oligonucleotide probe. Any source of
20 nucleic acid, in purified or nonpurified form can be utilized as the starting nucleic acid or acids, if it contains, or is suspected of containing, the target nucleic acid sequence. The target nucleic acid can be only a fraction of a larger molecule or can be present
25 initially as a discrete molecule. Additionally, the target nucleic acid may constitute the entire nucleic acid or may be a fraction of a complex mixture of nucleic acids.

30 The method of this invention requires formation of a hybrid between an oligonucleotide primer (referred to herein as the oligonucleotide probe) and the target nucleic acid sequence. Probes of relatively short length (e.g. 10-100 nucleotides) are preferred in that they can be chemically synthesized. The probe can consist of

interest. Similarly, amplification primers that are free in solution can be extended and provide incorporation at positions other than the position of interest. Various methods obvious to those skilled in the art of molecular biology are available for removing unincorporated nucleotides and primers. However, since we desire a method that is rapid and automatable, the preferred form of separation is one utilizing attachment of the amplified nucleic acid product to a solid support with subsequent washing steps. An avidin-biotin system is preferred.

The template may be RNA or DNA, and may be double or single stranded. If double stranded, it is necessary to denature the strands to allow hybridization between the template strand and the oligonucleotide probe. Methods for this denaturation and subsequent hybridization step are well known to those skilled in the art of sequencing. However, since it is well known that formation of the hybrid between the oligonucleotide probe and the nucleic acid strand containing the target nucleic acid sequence can be inhibited by the complementary, non-template strand, the preferred method is to physically separate the template and the non-template strand after a denaturation step. The template strand can either be the strand present on the solid support (Figure 2), or a strand that is free in solution (Figure 3).

By design of an appropriate probe and utilizing the appropriate nucleic acid strand as template, essentially all nucleotide positions, even those at the end of a linear nucleic acid molecule can be assayed (Figure 4). In practice, since amplification from a complex nucleic acid mixture will at times give several different amplification products, the preferred method is to

nucleotide addition by a DNA polymerase. For example, cleavage of DNA with many restriction enzymes generates 5' overhangs that are substrates for DNA polymerases. Also, there are 3' exonucleases that remove 3'

- 5 nucleotides from double-stranded DNA, producing molecules with 3' recessed strands and 5' overhanging strands.

The hybridizing and extending steps can be performed in solution or in solid phase reactions. The detection
10 can also be in solution, after attachment to a solid phase, or after passing through a gel such as acrylamide or agarose. However, as previously mentioned, the first two methods are preferred for they avoid the time-consuming gel assay. Without a gel assay, it is
15 necessary to separate the unincorporated labeled chain-terminators after the elongation step. Note that in the present invention, it is not necessary to wash away the excess oligonucleotide probe that did not hybridize, since the unextended probe does not contain a label.

- 20 There are four general forms of such separation: (1) immobilizing the elongated probe or hybrid selectively (e.g. by attaching to a binding segment on the analyte strand or on the probe) and separating away unincorporated, labeled nucleotide substrate together
25 with sample polynucleotides that probe did not bind to; (2) immobilizing the elongated probe or hybrid non-selectively with other polynucleotides and separating away the unincorporated, labeled nucleotide substrate; (3) separating the unincorporated, labeled nucleotide
30 substrate without immobilizing the elongated probe or hybrid, and (4) inactivating the label associated with unincorporated nucleotide substrate such that it is no longer detectable by the assay method employed.

present method should be independent of the binding system used to attach detectable label to the modified nucleotides during the detection step.

Crucial to this invention, are the chain-terminating, detectably labeled nucleotide substrates. Detectably labeled does not mean that the detectable signal must be present at the time of incorporation. The fluorescent substrates described below require activation. Detectably labeled does not necessarily mean that the nucleotide substrates carry a reporter such that there is not only the ability to detect the label, but also to identify the nucleotide. If only one nucleotide is present in the reaction, then detection of incorporation is sufficient for identification. The modified dideoxy-nucleotide substrates described in Prober et. al (EP-A 252683) or the DyeDeoxy terminators (a trademark of Applied Biosystems, Inc., Foster City, California) are examples of chain-terminating detectably labeled nucleotide substrates. However, unlike sequencing using fluorescently labeled chain-terminating nucleotides, there is essentially no requirement in this method that each of the modified nucleotides have a similar mobility shift when run on a sequencing gel. In the preferred embodiment, four chain-terminating nucleotides that are distinguishably labeled are present in each reaction. The need for four different labels is eliminated if the number of reactions per sample are increased (Figure 6). Unlike reports in the prior art, all four chain-terminating nucleotides may be present in the initial reaction, but only one must be detectably labeled. Unlike nucleic acid sequencing using chain terminators, the chain-elongating dNTP substrates are not a component of the reaction of the present invention.

The present invention is further illustrated by reference to Figures 1-6.

In Figure 1a, an analyte strand (An) contains a nucleotide position of interest (N), the identity of which is to be determined by the assay, is defined as the first base of the analyte nucleic acid strand which is beyond the 5' end of the target nucleotide sequence in the 3' to 5' direction. A probe polynucleotide is produced as a reagent having a binding region complementary to the target nucleotide sequence (TNS). In this particular embodiment, the probe polynucleotide consists only of that complementary sequence; in other embodiments, the probe is extended in the 5' direction in a manner that does not interfere with the recognition and complementary base pairing to the target nucleotide sequence. The diagram in Figure 1a illustrates the double stranded nucleic acid region which forms when the probe binds to analyte strand An by complementary base pairing to the target nucleotide sequence TNS.

By contacting the double stranded region shown in Figure 1a with a DNA polymerase specific therefore, the 3' end of the probe will be utilized as a primer and elongated opposite the analyte strand An which serves as a template for nucleotide incorporation. As illustrated in Figure 1b, the nucleotide incorporated (N*) will be complementary to the nucleotide position of interest (N). In all illustrations, the * symbol is used to illustrate a detectable label attached to the nucleotide. The enzyme, primer and nucleic acid analyte are chosen together such that a nucleotide complementary to the target nucleotide of interest is incorporated. For example, if analyte strand An is DNA, then a reverse transcriptase, a primer dependent prokaryotic DNA polymerase (e.g. the Klenow fragment of *E. coli* DNA

streptavidin complex, and rinsing away the unbound material. The two strands are then denatured (e.g. by addition of NaOH) and only the template strand is retained for the reaction. In Figure 2 the immobilized
5 template strand is rinsed, while in Figure 3 the soluble, eluted strand is used as template after neutralizing the NaOH solution. The probe oligonucleotide is then hybridized to the template strand and the hybridized probe is elongated by addition
10 of a single, chain terminating nucleotide. The enzyme utilized in the reaction is a DNA polymerase such as reverse transcriptase and all four chain terminating nucleotides may be present, although only one must be detectably labeled. The unincorporated nucleotides are
15 removed from the reaction by washing. Note that in Figure 3, the template strand was not previously immobilized, so the probe oligonucleotide can now be captured onto solid support for efficient washing. The nature of the label present on the elongated primer may
20 be measured directly after efficient removal of the unincorporated substrate. That is, the primer may still be bound to the solid support, either directly as shown in Figure 3 or indirectly through the hybrid formed with the analyte strand (Figure 2 without the final
25 denaturation step). For the particular brand of streptavidin coated magnetic beads used in our examples, the labeled primer is released from the beads after heating in the presence of formamide and EDTA. The magnetic beads do not interfere with standard gel
30 electrophoresis although they are loaded into the sample well along with the sample. If the sample is assayed through a capillary, then the beads may obstruct flow and should be removed.

Conclusion: Since the other nucleotides were not detected, only T is present at the nucleotide of interest. No other reactions are required.

5

Method 2

All possible nucleotide substrates are present in each reaction but perhaps only 2 can be detectably labeled such that they are distinguishable from each other.

10

1st Reaction: provide substrates ddATP*, ddGTP*, ddCTP, ddTTP.

Result: Only ddATP* is incorporated and detected.

15 Conclusion: T is present at the nucleotide position, and C is not present. But do not know if either G or A are present.

2nd Reaction: provide substrates ddCTP*, ddTTP*, ddATP, ddGTP.

20 Result: There was no detectable incorporation.

Conclusion: G or A are not present at the position of interest.

(To provide evidence to support this further, one could use the same substrate mixtures except monitor the incorporation using the complementary strand as the anlyate and the different, but appropriately positioned primer.)

25

Method 3

30 All possible nucleotide substrates are present in each reaction but perhaps only one label is available for substrate labeling (e.g., the same as when radioactively labeled ddNTP's are utilized).

2. To illustrate that a single fluorescently labeled nucleotide can be incorporated in the assay using a commercially available enzyme preparation.

3. To illustrate that the unincorporated labeled
5 substrate can be efficiently removed without time-
consuming centrifugation or column chromatography.

4. To illustrate the use of a DNA strand labeled at the 5' end with Biotin and bound to a solid support as the analyte strand.

10 5. To illustrate the ability to distinguish between
three DNA samples by incorporation of an A (wildtype
allele), a G (mutant allele), or both A & G
(heterozygote) as the complementary nucleotide opposite
the nucleotide position of interest.

15 6. To illustrate that the fluorescent nucleotide
substrates can be detected and distinguished on the
Genesis 2000 DNA analysis unit either by gel
electrophoresis or by passing the sample through a
capillary.

20

Definition of the Nucleotide Position of Interest:

In this example, the nucleotide position of interest is that of the lower strand at nucleotide position 5430 of the mouse RNA polymerase II largest subunit gene as described by publication in the GenBank database, accession M12130 for the locus RO:Musrpolii2. A 602 nucleotide portion of this sequence from nucleotide 4915 to 5517 is illustrated in its double stranded form in Figure 8, with the nucleotide position of interest for this example being at position 5430 on the lower strand (occupied by a bold-faced T in the sequence of the Wildtype allele which is shown in this Figure 8).

starting biological materials are obtained from J. Corden and are as described in Bartolomei and Corden, *Molec. and Cell. Biol.* 7:586-594, 1987. The Wildtype and Mutant alleles are provided as bacterial strains
5 containing the recombinant plasmids pE26-4 and pE26-7 respectively. The biological sample designated as Heterozygous is obtained as a cell line A21. DNA of each of the recombinant plasmids is prepared by standard molecular biology procedures (described in Sambrook et
10 al., *Molecular Cloning: A Laboratory Manual* 1989), and genomic DNA is prepared from the A21 cell line as described in Corsaro and Pearson, *Somatic Cell Genet.* 7:603-616, 1981.

15 Amplifying a segment of DNA containing the nucleotide position of interest and the target nucleotide sequence:

As shown in Figure 8, the target nucleotide sequence and the nucleotide position of interest are within a 602 base pair segment of the RNA polymerase II gene. The
20 copy number of this segment is increased using exponential amplification, using DNA of each of the three biological starting materials described above. The oligonucleotide primers used for PCR amplification of the region of interest in the RNA polymerase II gene
25 are designated PCR amplification Primer 1 (5' CAGACATTTGAGAATCAAGTGAATCG 3') and PCR amplification Primer 2 (5' BCTCGGCTCTCAGGACCATAATCAT 3') where B=biotin (see Figure 8). They are synthesized by standard phosphoramidate chemistry on an Applied
30 Biosystems DNA synthesizer. For the biotinylated primer, the biotin moiety is added at the 5' end during synthesis as described in Cocuzza US patent 4,908,453. All such oligonucleotides used in this patent are prepared for the inventors by the Du Pont

stranded PCR amplification product contains a biotin moiety due to the biotin originally presnet on PCR amplification Primer 2. Thus, the separation is done by binding the biotinylated PCR amplification product to a streptavidin-coated solid support and rinsing away the non-biotinylated, PCR amplification Primer 1 and the unincorporated nucleotides. The support-bound PCR amplification product is then denatured using NaOH, the complementary, non-analyte strand is removed and the remaining analyte strand which is still bound to the solid support is rinsed and ready for the primer extension reaction. These steps are illustrated in the schematic drawing of Figure 2 and described below as steps 1-6.

1. Magnetic, streptavidin-coated beads from the Dynal corporation (Dynabeads M-280 Streptavidin, at 6 x 10⁸ beads/ml) are washed and resuspended at the same concentration in Triton Wash Solution [0.17% (w/v) Triton X-100, 100 mM NaCl, 10 mM Tris-HCl pH7.5, 1 mM EDTA] essentially as described in the Application Brief 25 for the Genesis 2000 DNA analysis system.

2. Approximately 20 µl of double-stranded DNA template, amplified using PCR amplification with one of the two primers labeled with biotin, are mixed with 20 µl of washed Dynabeads and incubated at 37°C for 30 minutes. This mixture is gently shaken intermitently in order to keep the magnetic beads in solution.

3. After this incubation, the tube containing magnetic beads and DNA is placed near a magnet to draw the beads to one side of the tube. After approximately four minutes of magnetization, the supernatant is removed.

4. The beads (with DNA bound) are then washed three times with TE buffer (10 mM Tris pH8, 1 mM EDTA) using

reaction. In this particular Example 2, it is as follows:

- 1 μ l of 125 μ M SF-ddGTP-505
- 1 μ l of 125 μ M SF-ddATP-512
- 5 0.5 μ l Invitrogen Reverse Transcriptase (10 μ / μ l)

10. The labeling reaction is at 42°C for 10 minutes, and then the reaction is placed on ice and 100 μ l of TE is added.

- 10 11. The sample is again magnetized for 4 minutes and the supernatant removed, followed by 3 washes of 100 μ l TE buffer (magnetization between each wash) to remove unincorporated nucleotides.

- 15 12. The final supernatant is removed and the magnetic beads (with DNA bound) are resuspended in 6 μ l FE solution (95% formamide, 25mM EDTA) and stored -4°C until further use.

20 Detection of the chain terminating nucleotide attached to the probe:

- 25 13. The sample from step 12 is diluted 1:16 fold further in FE containing crystal violet, for easier visualization in loading the sample and to get the sample in a reasonable concentration for detection by slab gel electrophoresis on the Genesis 2000 DNA analysis system (methods as described by the instrument documentation, with a few parameters described in more detail below).

- 30 14. "Lane Finding" for the Genesis detection system is performed manually using a primer fluorescently labeled at the 3' end prepared in advance using terminal transferase and a fluorescent ddNTP as substrate as described in Trainor and Jensen, Nucl. Acids Res. 16:11846, (1988) that is electrophoresed into each

described in Prober et al. (1988) *Science* 238, 336-341. In brief, the chain terminators are distinguished by a ratio of the measured fluorescence from two photomultiplier tubes (PMT). Each PMT value is

5 displayed as either a red or green mark on the output computer monitor, with a sample forming a peak as it passes by the excitory laser. (In the black and white Figures required for this patent application, the original color of each line of the sample peak is

10 indicated). Unlike the normal method of fluorescent base detection when multiple peaks of a sequencing reaction are being analyzed, the commercial Genesis 2000 software is unable to determine the identity of the fluorescent nucleotides (base call) in this application,

15 for only a few peaks are present in the lane. It is therefore necessary to prepare a one time calibration on the instrument by preparing a set of expected values for the fluorescently labeled chain terminators at various dilutions in FE (95% formamide, 25mM EDTA). It is

20 important to note that the commercial instrument is designed to have a non-linear response of the two PMTs when the voltage is too high. We experimentally determined that the ratio obtained for the green peak height to the red peak height for a given fluroescent

25 substrate is relatively invariant from experiment to experiment over the range of 0.1-9 volts. Thus the green to red ratio of a peak is only determined if the reaction samples are within this voltage range.

The result of such a calibration (+/- one standard

30 deviation) is shown in Figure 7a by two different assay methods. The samples are either electrophoresed on a urea-polyacrylamide gel by standard gel electrophoresis procedures for the Genesis 2000, or syringe-loaded into a single, empty capillary (Part # TSP530700 from

and Het peak) are rerun at lower dilution (since the voltage of two of them are originally too high as shown in Figure 10a). The resulting sample peaks are displayed in Figure 10b with a smaller display window for easier measurement. In this example, the measured green/red ratios are as follows:

WT = 1.55

Mutant = 2.5

HET = 1.9

10 A comparison of these values to the calibration shown in Figure 7a illustrates that these ratios correspond to the expected incorporation of SF-ddATP-512 when the Wildtype allele is the source of the analyte strand, incorporation of SF-ddGTP-505 when the Mutant
15 allele is the source of the analyte strand, and a mixture of both fluorescent nucleotides in approximately equal proportions when the analyte strand is derived from a heterozygous source (i.e. approximately equal number of Wildtype and Mutant analyte strands).

20

Identifying the nucleotide of interest as the nucleotide complementary to the chain terminating nucleotide which is added:

17. The nucleotide at the position of interest is
25 the nucleotide complementary to the nucleotide that is incorporated.

Therefore, the conclusion for the three samples of this example are as expected:

The reaction performed on the Wildtype allele
30 indicates that it does contain a thymidine (T) on the lower strand at nucleotide position 5430 of the mouse RNA polymerase II largest subunit gene, for the nucleotide incorporated is SF-ddATP-512. The reaction performed on the Mutant allele indicates that it does

Definition of the Target Nucleotide Sequence:

In this example, the target nucleotide sequence (TNS) is chosen as the 21 nucleotide sequence
5 (5'ATGTAGAGGGCAAGCGGATCC3') that immediately flanks the nucleotide of interest such that the nucleotide position of interest is the next contiguous nucleotide in the 3' to 5' direction on that nucleic acid strand (see also Figure 1f).

10

The oligonucleotide probe:

In this example, the oligonucleotide probe consisted of the 21 nucleotide sequence 5' GGATCCGCTTGCCCTCTACAT 3' (probe B of Figures 8 and 9), and is perfectly
15 complementary to the target nucleotide sequence defined above. Synthesis and purification is as described in Example 1.

Starting biological sample:

20 In this example, the claimed method will be illustrated using two of the same starting biological samples as described in Example 1: that of the Wildtype and Mutant. They are prepared as described in Example 1.

25

Amplifying a segment of DNA containing the nucleotide position of interest and the target nucleotide sequence:

The region of interest is amplified from the Wildtype and Mutant samples using methods as described
30 in Example 1 with PCR amplification Primer 1 and PCR amplification Primer 2, except in this Example 2, the PCR amplification Primer 1 is biotinylated at the 5' end and the PCR amplification Primer 2 is not.

EXAMPLE 3

Aim:

1. To illustrate that under the conditions of Examples 1 and 2, that in some cases, the wrong nucleotide will be incorporated if the correct nucleotide is missing from the reaction (i.e. the problem with many of the assays discussed in the prior art is that of significant misincorporation in reactions where the correct nucleotide is not provided.

For this example, the same two reactions as described in Example 2 are performed with the following exceptions:

a) For the reaction with the Wildtype allele in step 9 the SF-ddTTP-526 is omitted and the only fluorescent substrate in the reaction is 1 μ l of 125 μ M SF-ddCTP-519. (Unlabeled ddTTP is also absent in the reaction).

b) For the reaction with the Mutant allele in step 9 the SF-ddCTP-519 is omitted and the only fluorescent substrate in the reaction is 1 μ l of 125 μ M SF-ddTTP-526. (Unlabeled ddCTP is also absent in the reaction).

The results shown in Figure 12 illustrate that for the Wildtype allele (upper panel), there is no significant misincorporation of SF-ddCTP-519 as a complementary base for the adenine (A) present as template for the primer extension. This is true although the SF-ddCTP-519 is present at a higher concentration than in Example 2. No conclusions can be made with respect to whether A or G is incorporated, for these nucleotides although present, are not fluorescently labeled in the reaction. The lower panel of Figure 12 illustrates a significant level of misincorporation of SF-ddTTP-526 as a complementary base

discriminatory values given in Figure 7b). In these lanes, as in other lanes of the experiment, there is essentially no peak of unincorporated nucleotides present in the sample.

5

EXAMPLE 5

Aims:

1. To illustrate the use of a nucleic acid strand that is not bound to a solid support as the analyte strand.

10

2. To practice the method of this invention on a totally different biological sample than that used in Examples 1-4.

15 Definition of the Nucleotide Position of Interest:

In this example, the nucleotide position of interest is that of the lower strand, occupied by a circled G on Figure 14. The nucleotide sequence shown is a portion of the Wildtype A1 gene of maize (Schwarz-Sommer et al., EMBO J. 6:287-294 (1987)).

20

Definition of the Target Nucleotide Sequence:

In this example, the target nucleotide sequence (TNS) is chosen as the 21 nucleotide sequence (3'GACGAACTCCTAGCTCATCAC5') that immediately flanks the nucleotide of interest such that the nucleotide position of interest is the next contiguous nucleotide in the 3' to 5' direction on that nucleic acid strand.

25

30 The oligonucleotide probe:

In this example, the oligonucleotide probe consists of the 21 nucleotide sequence 5' CTGCTTGAGGATCGAGTAGTG 3' (Primer C of Figure 14), and is perfectly complementary to the target nucleotide sequence defined

NaOH solution is carefully neutralized by addition of a few microliters of 0.5M HCl, monitoring the pH of the solution using pH paper. To this neutralized DNA sample (vol. aprox. 23 μ l), the following addition is made:

- 5 8.0 μ l of 5X RT buffer (Invitrogen)
- 2.8 μ l of 1% Triton X-10
- 0.5 μ l of 1 mM ddATP (unlabeled)
- 0.5 μ l of 1 mM ddTTP (unlabeled)
- 4.0 μ l of 6.6 μ M Biotinylated nested primer
- 10 8. The sample is incubated 95°C for 2 minutes, 37°C
- 10 minutes, and then placed on ice.
9. The following additions are made:
- 2 μ l of 125 μ M SF-ddCTP-519
- 2 μ l of 125 μ M SF-ddGTP-505
- 15 1 μ l of Reverse Transcriptase (Invitrogen 10
- μ /ml)
10. The sample is incubated 42°C 10 minutes.
11. 15 μ l of Dynabeads (prepared as in step 1) are
- added and followed by a 37°C incubation for 15 minutes
- 20 with intermittent shaking. This is to promote binding of
- the nested, biotinylated primer (containing fluorescent
- label from the primer extension reaction).
13. The sample is magnetized for 4 minutes and
- unincorporated nucleotides present in the supernatant
- 25 are removed.
14. The final bead pellet is washed 3 times with 100
- μ l TE (magnetizing each time to remove the buffer).
15. The final bead pellet is resuspended in 5 μ l of
- FE (95% formamide, 25 mM EDTA).
- 30 16. 2 μ l of this sample along with 1 μ l of a
- smaller, control primer (Std) are heated for 2 min 95°C,
- before loading on a urea-polyacrylamide gel and
- electrophoresis on the Genesis 2000.

8.0 μ l of 5X Sequenase buffer (200 mM Tris pH 7,
100 mM MgCl₂, and 250 mM NaCl)
2.8 μ l of 1% Triton X-100
0.5 μ l of 1 mM ddATP (unlabeled)
5 0.5 μ l of 1 mM ddTTP (unlabeled)
4.0 μ l of 6.6 μ M Biotinylated oligonucleotide
probe (primer C)

c) In step 9, 1.5 μ l of 100 mM Dithiothreitol is
added (in addition to the fluorescent substrates SF-
10 ddCTP-519 and SF-ddGTP-505), and 1 μ l of 13 units/ μ l
Sequenase Version II enzyme (a modified T7 DNA
polymerase: US Biochemical Corp. US Patent 4,795,699)
instead of the reverse transcriptase enzyme.

d) In step 10, the labeling reaction is at 37°C for
15 10 minutes.

The results of Example 6 are shown in Figure 16. It
is seen that a single peak of incorporation appears,
suggesting that the Sequenase II enzyme can also be used
for the practice of this invention with no significant
20 3'-5' exonuclease activity. The green/red ration (=1.7)
of this peak is as would be expected for a DNA sample
that is heterozygous. That is, the calibration graph of
Figure 7c indicates that the value of 1.7 is
approximately equal distance between the expected values
25 for incorporation of SF-ddCTP-519 (green/red
approximately 0.9) and that for incorporation of
SF-ddGTP-505 (green/red approximately 2.4). Note that
in this example, SF-ddATP-512 that gives a green/red
ratio of approximately 1.6 is not included in the
30 reaction, thus the 1.7 ratio does not indicate the
addition of an adenine.

In conclusion, in Example 6 it is determined that
the nucleotide position of interest in the sample is

The chain-terminating nucleotide which extended the probe at the position complementary to the nucleotide of interest is detected and determined as shown in Example 1. The nucleotide of interest at position 500 is
5 identified as the nucleotide complementary to the chain-terminating nucleotide which extended the probe in the labeling reaction. The presence and nature of a polymorphism can be determined by comparing the samples tested.

10

From the foregoing description, one skilled in the art can easily ascertain characteristics of this invention, and without departing from the spirit and scope thereof, can make various modifications of the
15 invention to adapt it to various uses and conditions.

20

25

30

(B) TELEFAX: 302-892-7949

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTGCTGCCCCG ACAACAGCAA T

21

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATTGCTGTTG TCGGGCAGCA G

21

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CAGACATTTG AGAATCAAGT GAATCG

26

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CACTACTCGA TCCTCAAGCA G

21

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CTGCTTGAGG ATCGAGTAGT G

21

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATTGCTGTTG TCGGGCAGCA G

21

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 43 base pairs
 - (B) TYPE: nucleic acid

- (ii) MOLECULE TYPE: DNA (genomic)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ATTGCTGTTG TCGGGCAGCA GG

22

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 43 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GGATCCGCTT GCCCTCTACA TCCTGCTGCC CGACAACAGC AAT

43

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 43 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

ATTGCTGTTG TCGGGCAGCA GAATGTAGAG GGCAAGCGGA TCC

43

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

ATTGCTGTTG TCGGGCAGCA GGATGTAGAG GGCAAGCGGA TCC

43

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GGATCCGCTT GCCCTCTACA TC

22

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 43 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GGATCCGCTT GCCCTCTACA TTCTGCTGCC CGACAACAGC AAT

43

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 45 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

TGCTGTTGTC GGGCAGCAGA AA

22

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 45 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GGATCCGCTT GCCCTCTACA TTTCTGCTG CCCGACAACA GCAAT

45

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 603 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CAGACATTTG AGAATCAAGT GAATCGTATT CTCAATGATG CTCGAGACAA AACTGGCTCC 60

TCTGCACAGA AATCCCTCTC TGAATATAAC AACTTCAAGT CTATGGTGGT GTCTGGAGCC 120

AAGGGTTCCA AGATCAACAT CTCCCAGGCA AGATGCTTCA TTTTCCAGAT ATGTGGCCTA 180

TACCAGAGTT TGTAAGAGG ATGGTATGTA CATGTTTTGG TGTGAGGAAA GATGGAAAAA 240

ATAGTAGGGA ATTGTCACCA CCACCACCAC TGCTGCAGTG TCATGGCTTG AAACAAGATT 300

CACTCACGTG TAAAAGACCT TTTTAAAAC AAAACAAAAC ATGGTTTTGC TGTGTAGCCC 360

CTTGGCTCCA GACACCACCA TAGACTTGAA GTTGTATAT TCAGAGAGGG ATTTCTGTGC 540

AGAGGAGCCA GTTTGTCTC GAGCATCATT GAGAATACGA TTCACTTGAT TCTCAAATGT 600

CTG

603

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

CCCCCCCCTTA GGTCATTGCT GTTGTCTGGGC AGCAGATGT AGAGGGCAAG CGGATCCCAT 60

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

ATGGGATCCG CTTGCCCTCT ACATTCTGCT GCCCGACAAC AGCAATGACC TAAGGGGGGG 60

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 295 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

CCTGCAGATG TACTTCGTGT CTAAACCCT GCGGAGAAG GCGCCCTGG CGTACGCGGC 60

GGAGCACGGC CTGGACCTGG TCACCATCAT CCCGACGCTC GTGGTCGGCC CGTTCATCAG 120

CGCGTCCATG CCGCCCAGCC TCATCACCGC GCTGGCGCTC ATCACGGGGA ACGCGCCGCA 180

CTACTCGATC CTCAAGCAGG TGCAGCTCAT CCACCTCGAC GACCTCTGCG ACGCCGAGAT 240

CTTCCTCTTC GAGAACCCGG CCGCGGCCGG GCGCTACGTC TGCTCCTCGC ACGAC 295

6. The method of Claim 1 wherein the probe is extended enzymatically.

7. The method of Claim 1 wherein the added chain
5 terminating nucleotide is determined by detecting the presence of a signal generator.

8. The method of Claim 1 wherein the nucleic acid
10 analyte is single stranded.

9. The method of Claim 1 wherein the nucleic acid analyte is immobilized on a solid support.

10. The method of Claim 1 wherein the probe is
15 immobilized on a solid support.

11. A kit for identification of a nucleotide of interest in a nucleic acid analyte, comprising:

- 20 a) a probe which comprises a primer sequence complementary to the nucleic acid analyte and capable of binding the nucleic acid analyte with sufficient specificity to form a stable hybrid adjacent to the nucleotide of interest;
- b) a plurality of reporter labeled chain
25 terminating nucleotide triphosphates; and
- c) a primer-dependent nucleic acid polymerase.

12. A method of identifying a nucleotide of interest present at a defined position in a nucleic acid analyte,
30 comprising:

- a) contacting the nucleic acid analyte with a probe such that annealing takes place adjacent to the nucleotide of interest;

20. The method of Claim 12 wherein the nucleic acid analyte is immobilized on a solid support.

21. The method of Claim 12 wherein the probe is
5 immobilized on a solid support.

22. A kit for identification of a nucleotide of interest in a nucleic acid analyte, comprising:

- a) a probe which comprises a primer sequence
10 complementary to the nucleic acid analyte and capable of binding the nucleic acid analyte with sufficient specificity to form a stable hybrid adjacent to the nucleotide of interest;
- b) at least one of reporter labeled chain
15 terminating nucleotide triphosphates; and
- c) a primer-dependent nucleic acid polymerase.

20

25

FIG. 1C

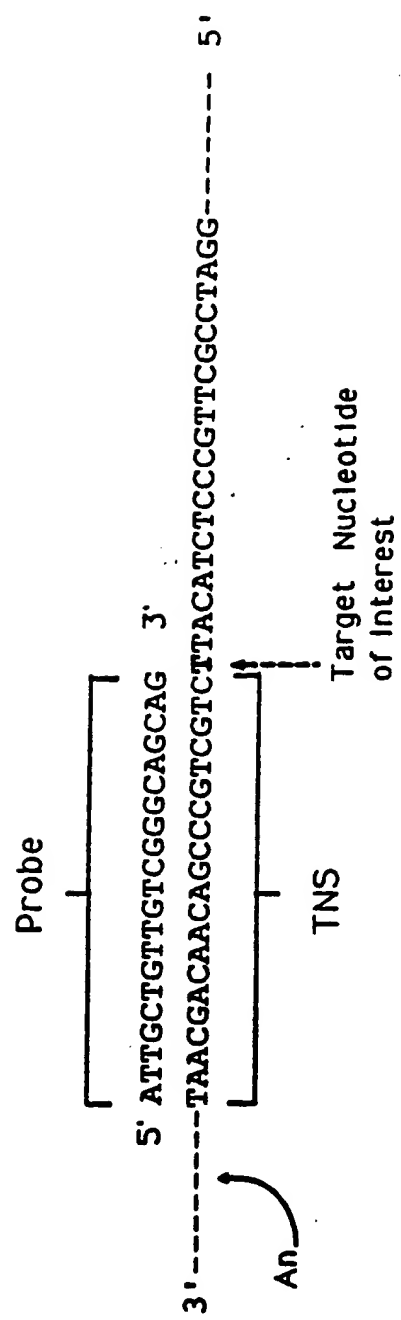


FIG. 1D

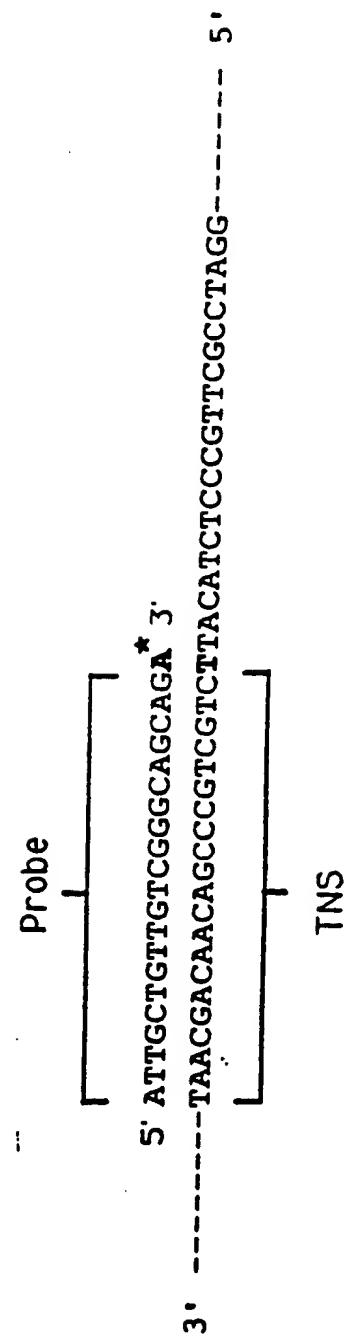


FIG. 1G

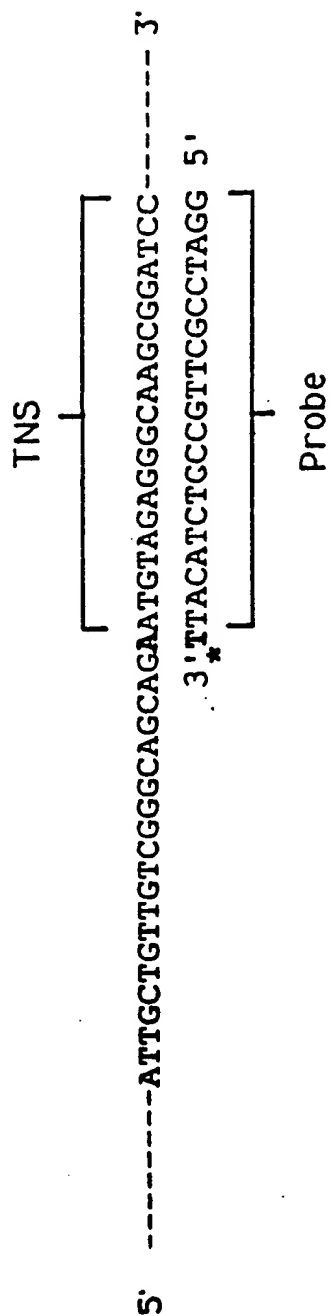
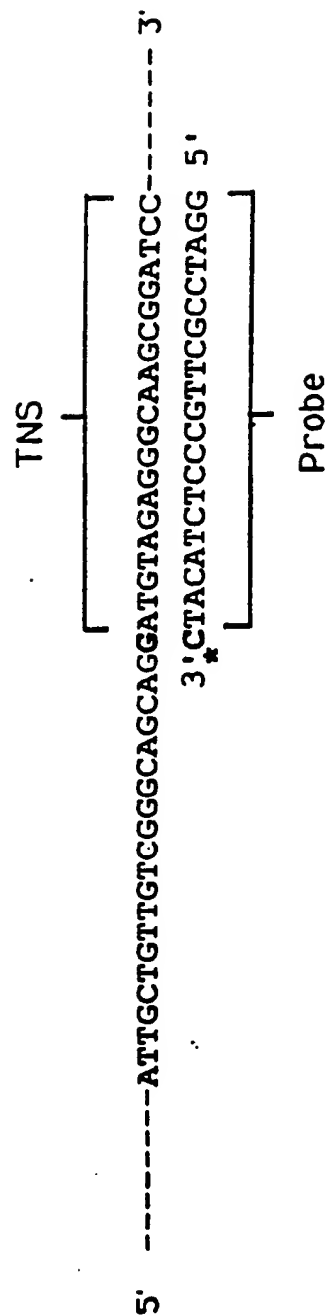


FIG. 1H



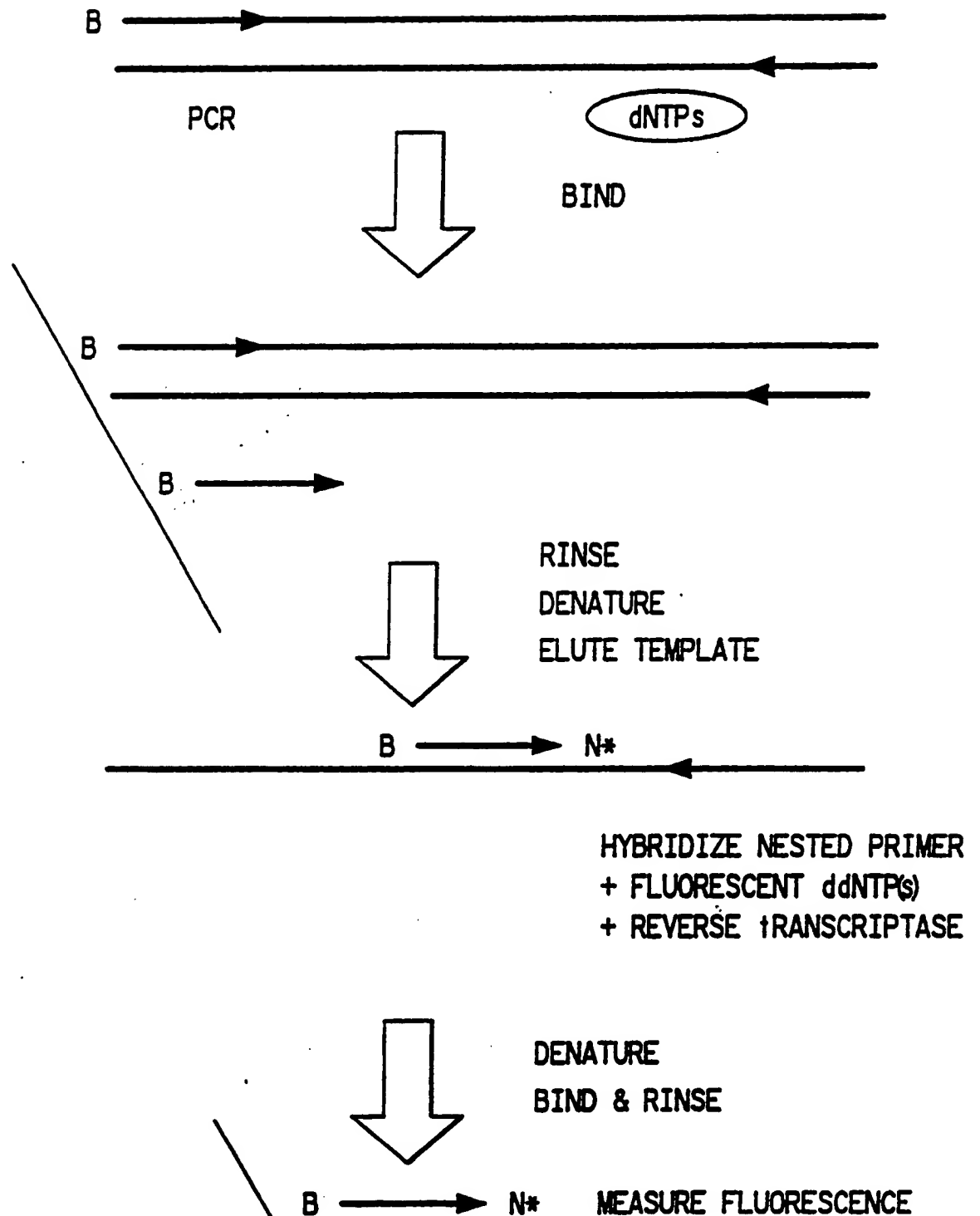
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FIG.3

FIG. 5B

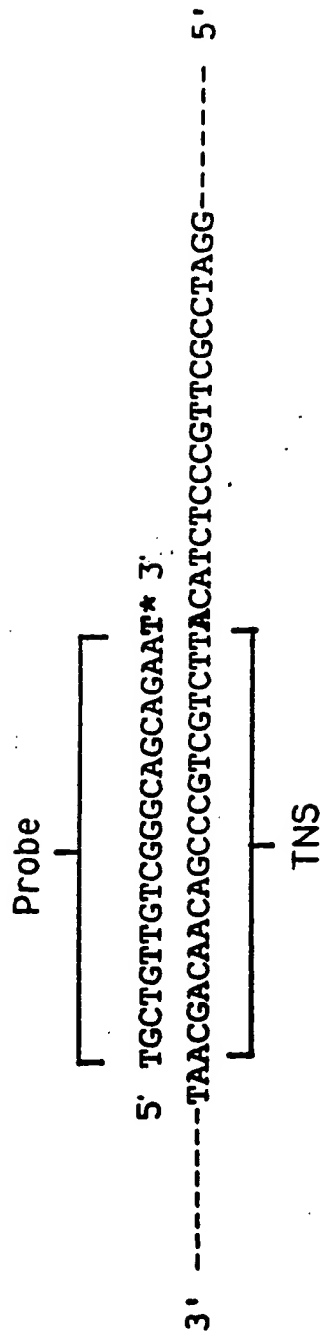


FIG. 5C

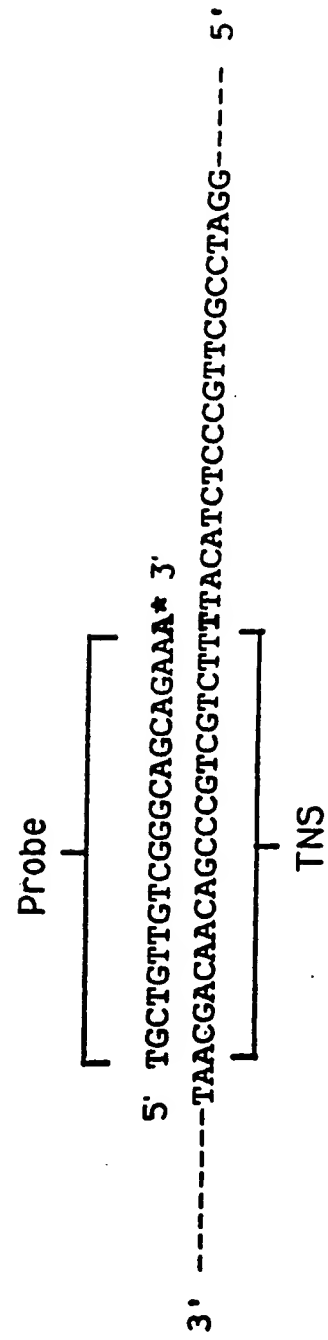
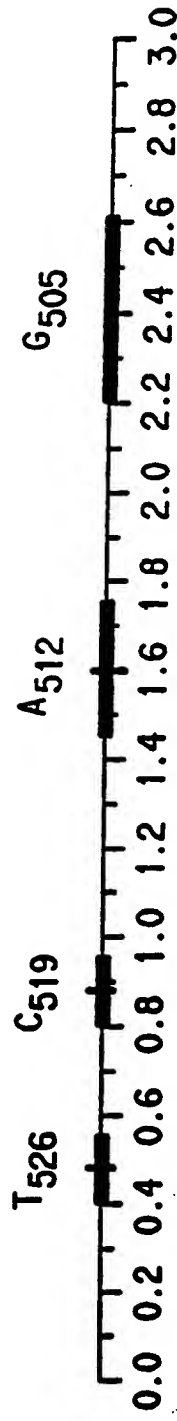


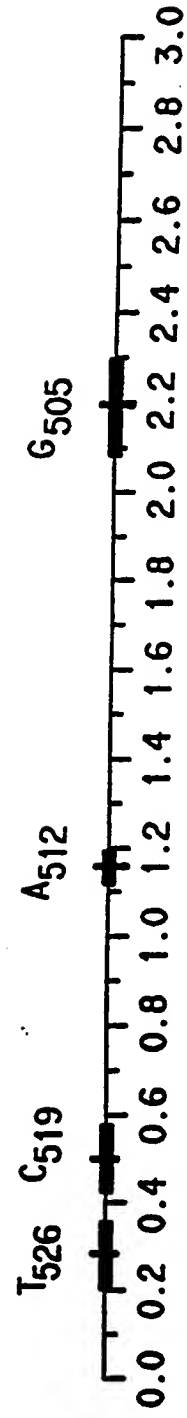
FIG. 7A

GREEN TO RED RATIO FOR FLUORESCENT NUCLEOTIDES

DETECTED USING GEL ELECTROPHORESIS:

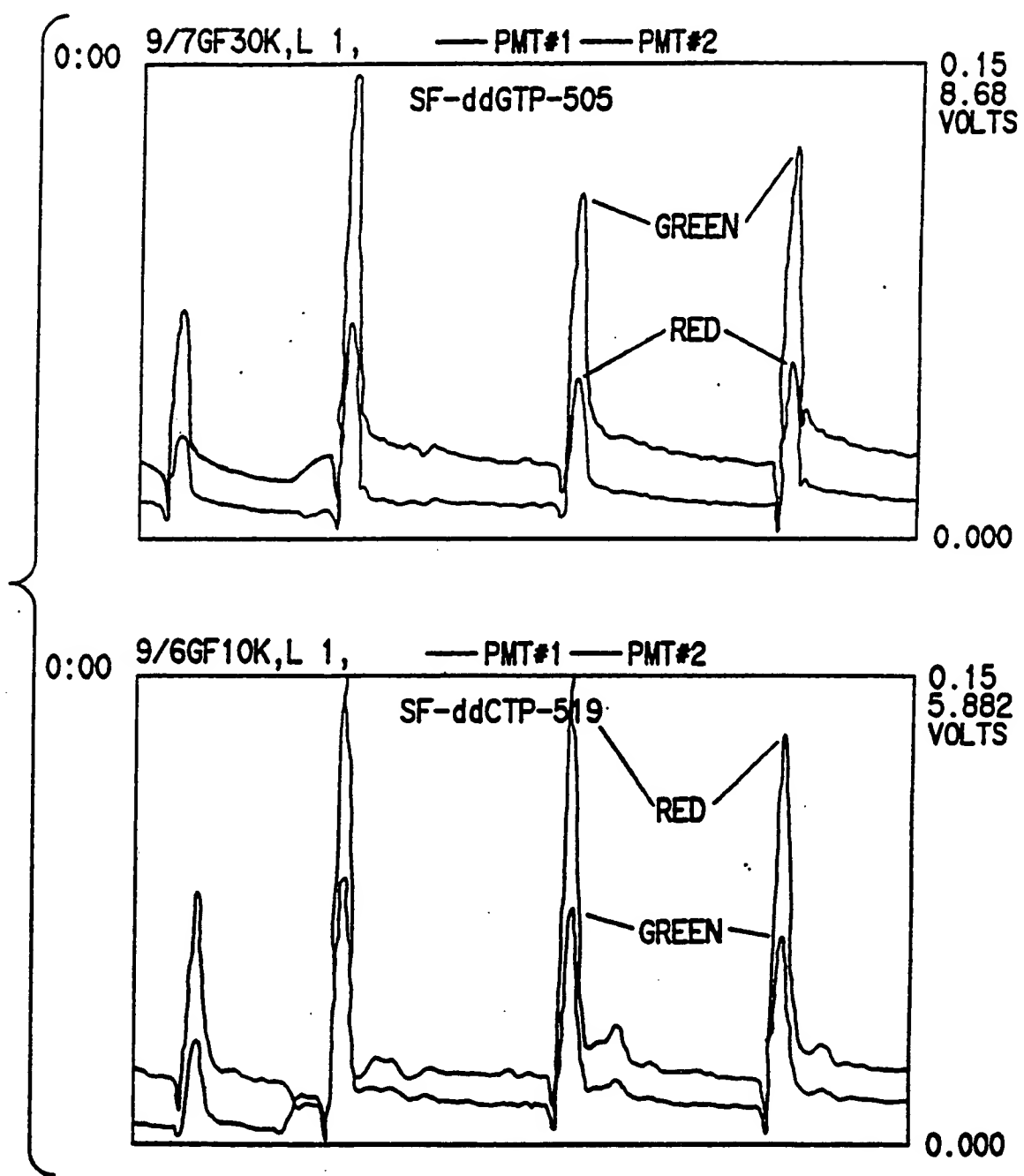


DETECTED IN CAPILLARY:

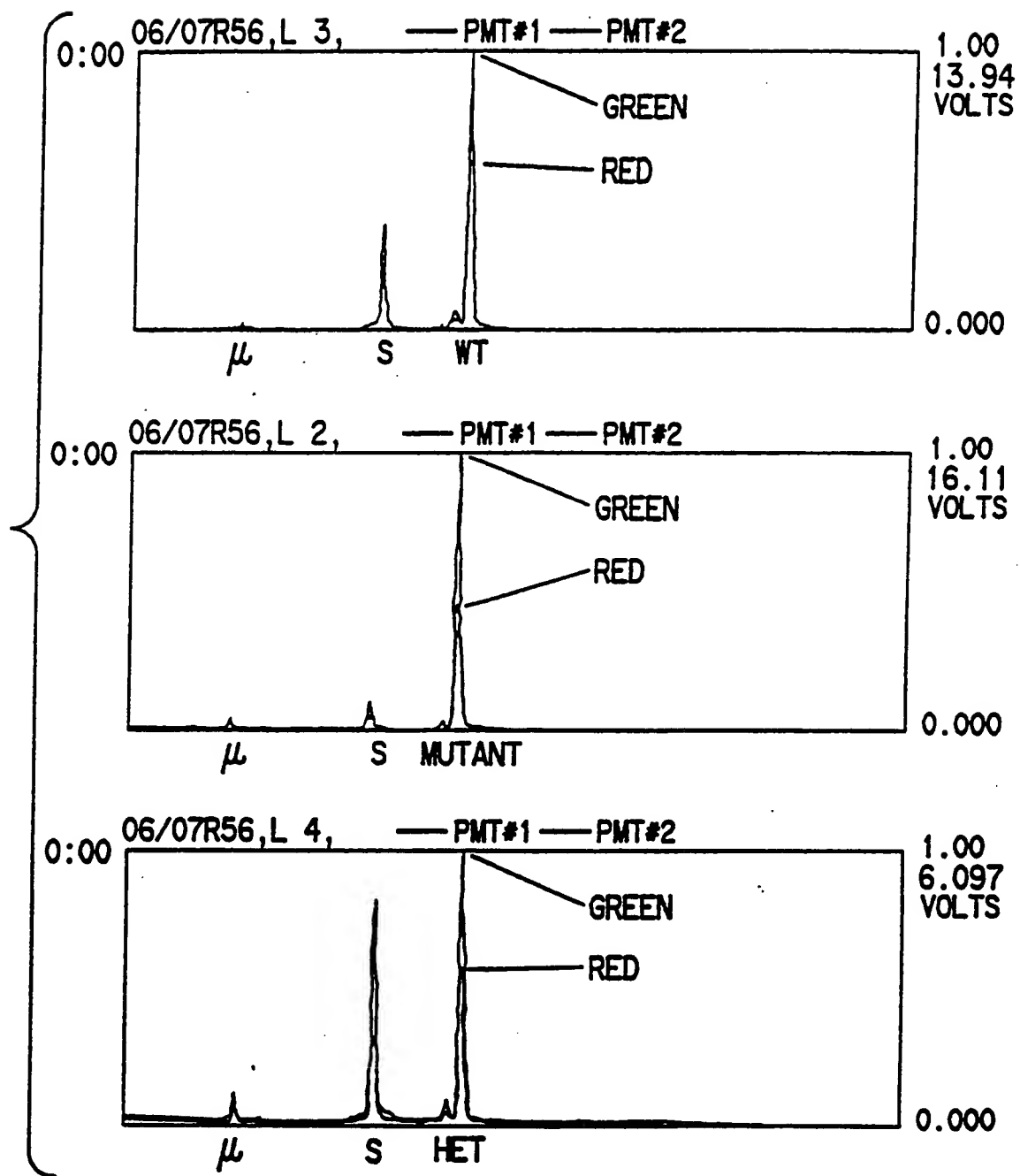


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FIG. 7C

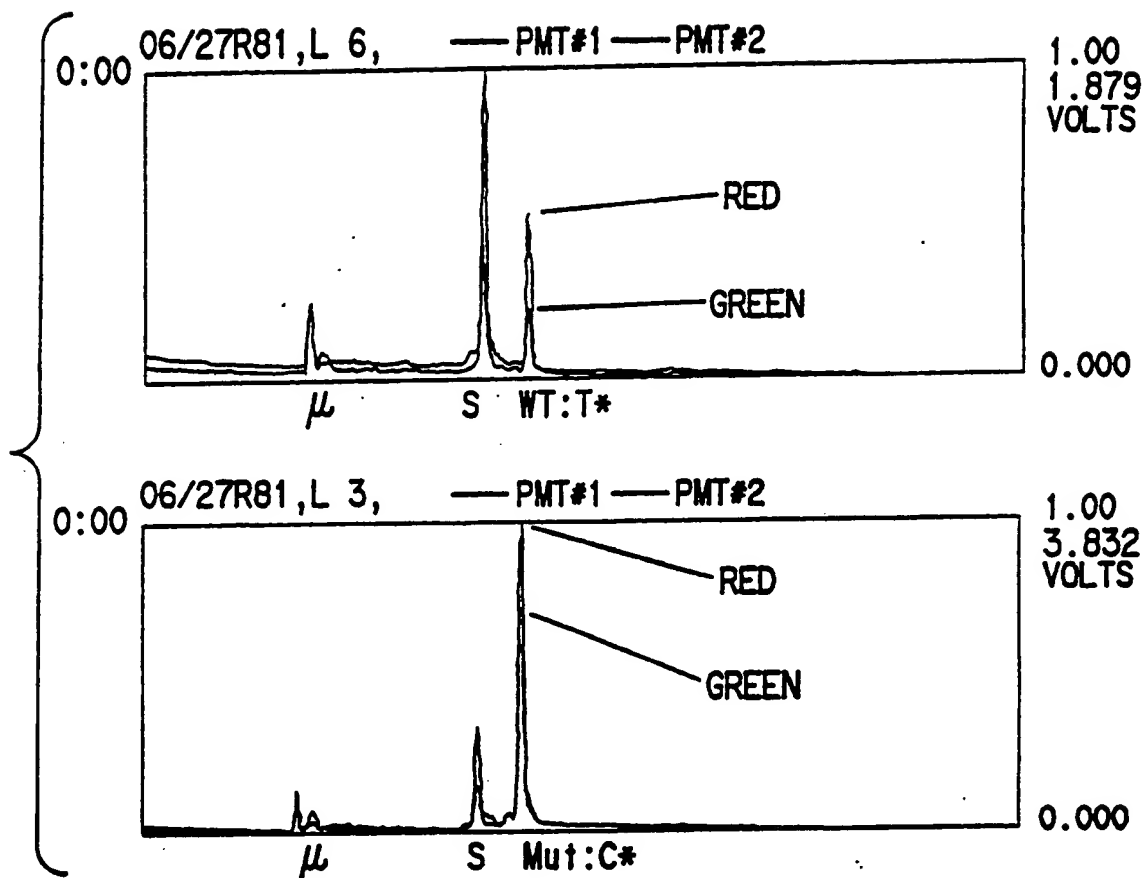


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FIG. 10A



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FIG. 11



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FIG. 13

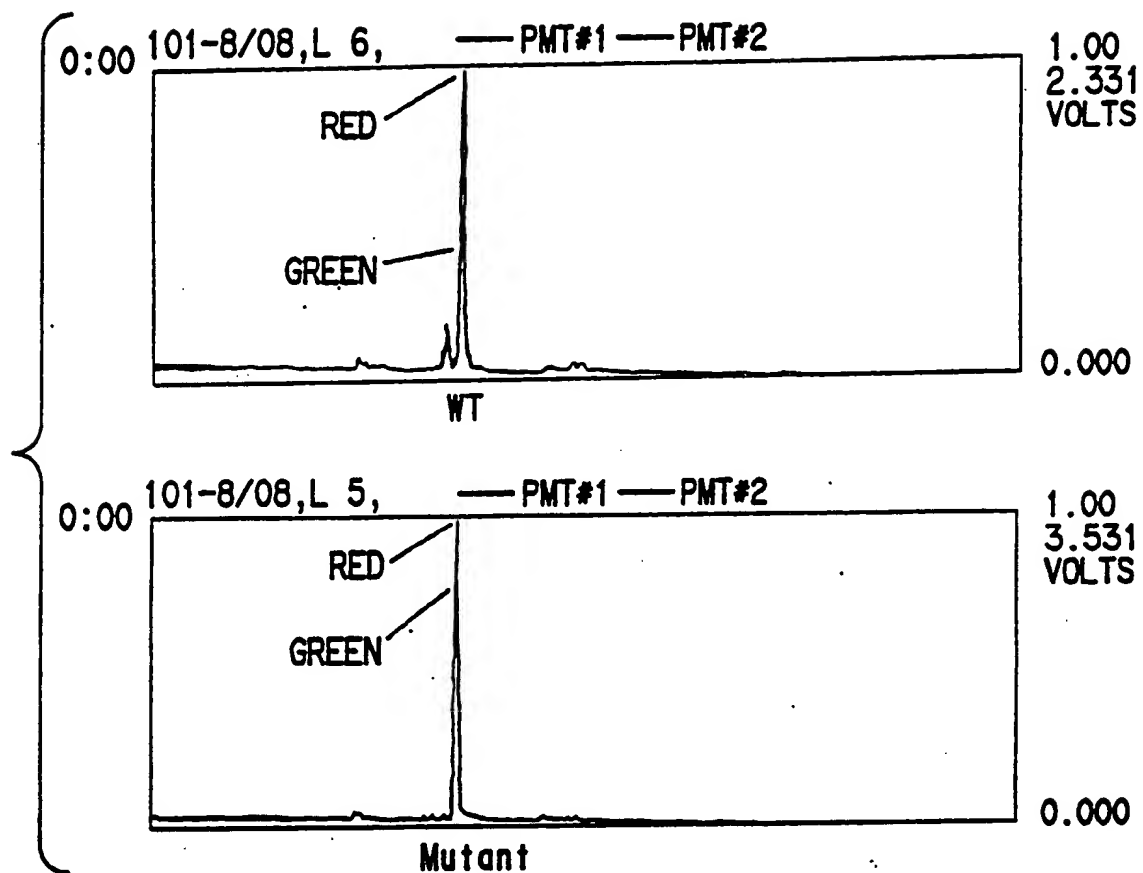
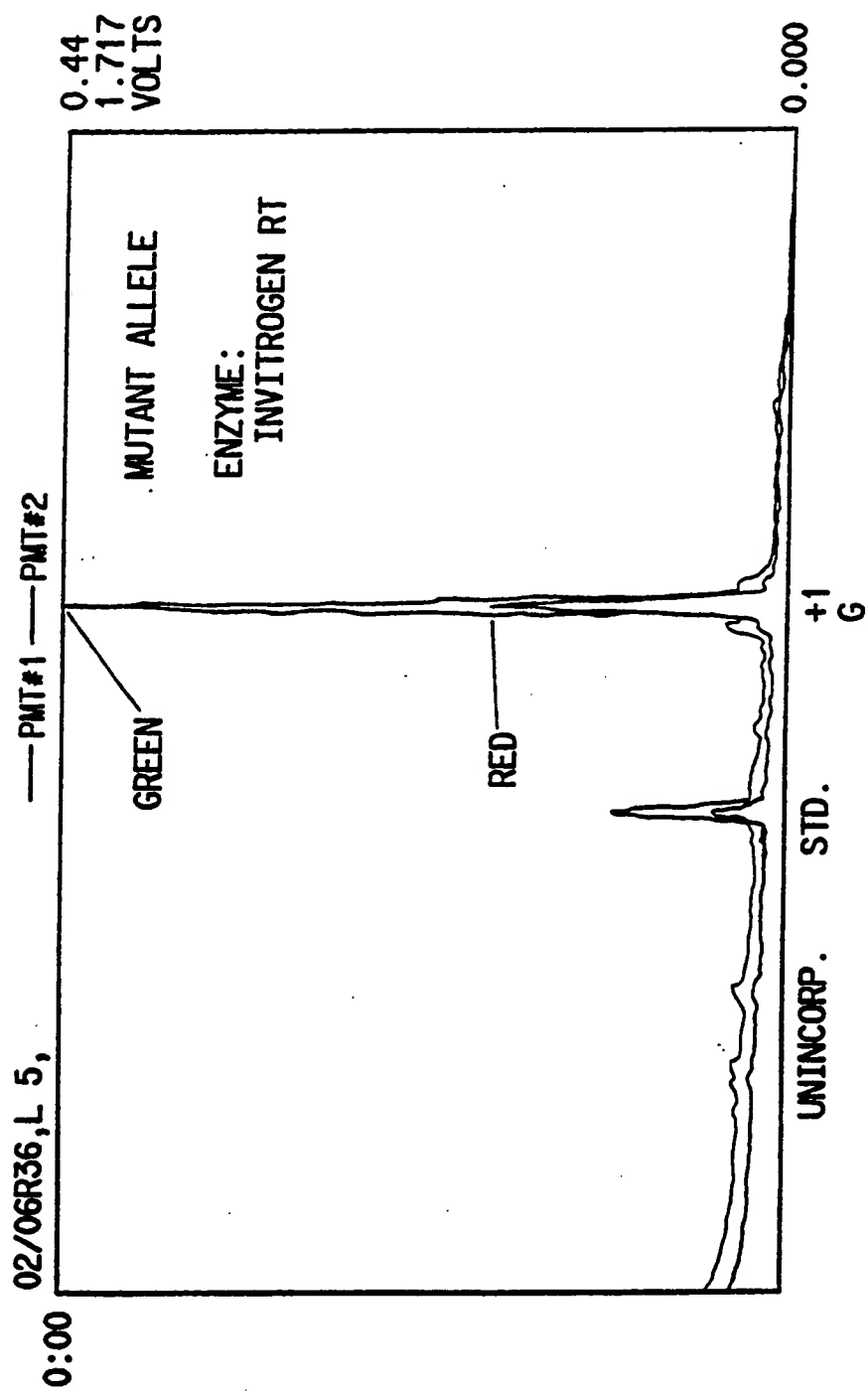


FIG. 15



I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)⁶

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12Q1/68

II. FIELDS SEARCHED**Minimum Documentation Searched⁷**

Classification System

Classification Symbols

Int.Cl. 5

C12Q

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched⁸**III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹**

Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	EP,A,0 412 883 (BERTIN & CIE) 13 February 1991 see the whole document ---	1-22
Y	NUCLEIC ACIDS RESEARCH. vol. 18, no. 12, 1990, ARLINGTON, VIRGINIA US page 3671; B. P. SOKOLOV: 'Primer extension technique for the detection of single nucleotide in genomic DNA' cited in the application see the whole document --- -/-	1-22

*** Special categories of cited documents: ¹⁰****"A"** document defining the general state of the art which is not considered to be of particular relevance**"E"** earlier document but published on or after the international filing date**"L"** document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)**"O"** document referring to an oral disclosure, use, exhibition or other means**"P"** document published prior to the international filing date but later than the priority date claimed**"T"** later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention**"X"** document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step**"Y"** document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.**"A"** document member of the same patent family**IV. CERTIFICATION**

Date of the Actual Completion of the International Search

18 JUNE 1992

Date of Mailing of this International Search Report

30.06.92

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

MOLINA GALAN E.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. US 9201691
SA 57791**

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for those particulars which are merely given for the purpose of information. 18/06/92

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0412883	13-02-91	FR-A- 2650840 AU-A- 6180190 WO-A- 9102087	15-02-91 11-03-91 21-02-91

WO-A-8909283	05-10-89	US-A- 4971903 AU-A- 3354889	20-11-90 16-10-89

WO-A-9106678	16-05-91	EP-A- 0450060	09-10-91
